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Detection of feline Coronavirus in effusions of cats with and without feline infectious peritonitis using loop-mediated isothermal amplification

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ABSTRACT

Feline infectious peritonitis (FIP) is a fatal disease in cats worldwide. The aim of this study was to test two commercially available reaction mixtures in a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay to detect feline Coronavirus (FCoV) in body cavity effusions of cats with and without FIP, in order to minimize the time from sampling to obtaining results.

RNA was extracted from body cavity effusion samples of 71 cats, including 34 samples from cats with a definitive diagnosis of FIP, and 37 samples of control cats with similar clinical signs but other confirmed diseases. Two reaction mixtures (Isothermal Mastermix, OptiGene Ltd.and PCRun™ Molecular Detection Mix, Biogal) were tested using the same primers, which were designed to bind to a conserved region of the FCoV membrane protein gene. Both assays were conducted under isothermal conditions (61 °C–62 °C). Using the Isothermal Mastermix of OptiGene Ltd., amplification times ranged from 4 and 39 min with a sensitivity of 35.3% and a specificity of 94.6% for the reported sample group. Using the PCRun™ Molecular Detection Mix of Biogal, amplification times ranged from 18 to 77 min with a sensitivity of 58.8% and a specificity of 97.3%.

Although the RT-LAMP assay is less sensitive than real time reverse transcription PCR (RT-PCR), it can be performed without the need of expensive equipment and with less hands-on time. Further modifications of primers might lead to a suitable in-house test and accelerate the diagnosis of FIP.

Feline coronavirus (FCoV), a member of the genus *Alphacoronavirus* of the subfamily *Coronavirinae*, family *Coronaviridae* within the order *Nidovirales* (de Groot et al., 2011), belongs to a group of enveloped, positive-sense RNA viruses that cause diseases in several species, such as severe acute respiratory syndrome (SARS) in humans or transmissible gastroenteritis (TGE) in pigs. Despite the high prevalence of FCoV infections in the cat population worldwide, only 5–10% of FCoV-infected cats develop the fatal disease feline infectious peritonitis (FIP) (Addie and Jarrett, 1992). This change of virulence of a harmless FCoV biotype that usually causes no clinical signs into the pathogenic variant is thought to be caused by mutations in the FCoV spike protein gene (Chang et al., 2012; Vennema et al., 1998). These mutations cause a change in tropism from enterocytes to macrophages, giving FCoV the ability to infect and effectively replicate within cells of the macrophage lineage and cause a lethal systemic disease with multi-organ involvement (Pedersen, 2009). The median survival time of cats with effusive FIP is only a few days (Ritz et al., 2007), and the diagnosis of FIP commonly leads to euthanasia, since to date, no treatment has been proven to be effective. Cats with FIP show nonspecific clinical signs such as fever, weight loss and anorexia, often accompanied by body cavity effusions and/or ocular and neurological signs. A definitive diagnosis of FIP *ante mortem* remains challenging, especially when no body cavity effusions can be detected (Hartmann et al., 2003). Presently, the gold standard for the diagnosis of FIP is considered to be immunostaining of FCoV antigen in macrophages within tissue lesions, a technique that requires invasive tissue collection (Kipar and Meli, 2014). In cats with FIP, FCoV can be detected by RT-PCR in cell-free body cavity effusions in more than 80% of the cases, while serum or blood samples often are negative (Doenges et al., 2017). For both immunostaining and RT-PCR, samples have to be sent to specialized laboratories, resulting in the delay of diagnostic results. This leads to further unnecessary testing for other diseases, to withholding necessary therapy of other treatable diseases, or to delayed euthanasia in cats suffering from severe signs of FIP. Therefore, a fast and simple point of care test would be very beneficial in the diagnostic process.

Loop-mediated isothermal amplification (LAMP) is a simple, rapid, and cost-effective nucleic acid amplification method (Notomi et al., 2000) and is already used for the detection of Coronaviruses in humans

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and several animal species (Hong et al., 2004; Nemoto et al., 2015). A set of four to six primers is used, that form products with self-hybridizing loop structures. By using a DNA polymerase with strand displacement activity, no melting or annealing steps are required, and amplification products of different lengths are formed at a constant temperature of 60–65 °C (Nagamine et al., 2002). Since LAMP reactions only require a simple heat block with constant temperature, and DNA amplification can be detected by fluorescence or color change, the method can be applied for point-of-care diagnostics (Surabattula et al., 2013).

The aim of this study was to test specificity and sensitivity of two commercially available reaction mixtures in a reverse transcription LAMP (RT-LAMP) to detect FCoV in body cavity effusions of cats with and without FIP, and to minimize the time from sampling to obtaining results.

This study included 71 cats that were presented to the Clinic of Small Animal Internal Medicine, LMU Munich, Germany. All cats included had body cavity effusions. In every cat presenting with body cavity effusions, FIP is a potential differential diagnosis. An earlier study showed that FIP is responsible for about 40% of effusions, while most of the remaining cases were caused by malignomas, cardiac insufficiency or purulent serositis (Hirschberger et al., 1995). The FIP group (n = 34) included cats with a definitive diagnosis of FIP by one or more methods: All effusions of cats with FIP tested positive for FCoV by RT-PCR by a commercial laboratory, and in 26/34 samples putative disease-causing mutations could be detected. The RT-PCR detection method has been described previously (Felten et al., 2017). In 25/34 cats FIP diagnosis was achieved by post-mortem examination, including full body necropsy with histopathological examination. Diagnosis of FIP was confirmed when typical histologic lesions where detected (surface-bound multi-systemic pyogranulomatous and fibrinonecrotic disease with venulitis with or without high-protein exudate). In 17/25 cats with full body necropsy immunohistological staining for FCoV-antibody was done on tissue sections and returned a positive result. Immunofluorescent staining of FCoV antigen in macrophages of thoracic or abdominal effusion was done in 20/34 cats, and all samples returned a positive result. A summary of the cases in the FIP group can be found in the supplementary Table 1.

Cats were included in the control group (n = 37) if they were definitively diagnosed with a disease other than FIP that explained the effusion. Cats of the control group suffered from neoplasia (n = 20), decompensated cardiac diseases (n = 12), inflammatory diseases (n = 2), such as bacterial peritonitis and pleurisy, or other diseases (n = 3). One cat had chronic thoracic chylous effusion of unknown origin and secondary fibroplastic pleurisy. In another cat, an end stage kidney disease caused effusion, and one cat had thoracic effusion after subcutaneous urethral bypass placement, which resolved after treatment. The diseases of the cats of the control group (n = 37) were definitively confirmed ante-mortem (n = 18) or at necropsy with histopathological examination (n = 19). Ante-mortem diagnosis was established by echocardiography for cardiac diseases (n = 8), and by cytology for neoplasia (n = 10). Immunofluorescent staining of FCoV antigen in macrophages of thoracic or abdominal effusion was done in 11/37 cats, with three positive and eight negative results. All effusions of the cats in the control group were tested for FCoV by RT-PCR, and all results were negative. The RT-PCR detection method has been described previously (Felten et al., 2017). A summary of the cases in the control group can be found in supplementary Table 2.

Body cavity effusion samples of all cats were obtained *ante mortem* with ultrasound guidance for diagnostic purposes. The use of samples for this study was approved by the Institutional Animal Care and Use Committee ('Ethikkommission des Zentrums für klinische Tiermedizin'), permission number 32-25-06-2014. Samples were stored at -80 °C in a 1.5 ml Eppendorf Safe-Lock microcentrifuge tube until assayed. All samples were centrifuged for 20 s at 15,000 × *g*. The supernatant of centrifuged thoracic and abdominal fluids was used for RNA extraction. When using fresh fluid samples, omission of the centrifugation step should be considered to include intact cells with a high viral burden (Pedersen et al., 2015). In thawed samples, cell integrity is lost and cell debris can be removed. Viral RNA was isolated using the commercial ZR Viral RNA KIT™ (Zymo Research Corp.) following the manufacturer's instructions. Briefly, 100 µl aliquots of samples were mixed with a buffer that facilitates viral particle lysis and allows for RNA adsorption onto the matrix of the Zymo-Spin™ Column. Then the RNA was washed and eluted with 15 µl of RNase free water. Extracted RNA aliquots were stored at −80 °C in an Eppendorf 1.5 ml Safe-Lock microcentrifuge tube until further processing.

The RT-LAMP primer design was assisted by the software PrimerExplorer (https://primerexplorer.jp/e/). Based on sequence analysis, the gene for the membrane protein (M) was selected as a target because it is highly conserved among FCoV strains. The DNA sequence from position 26,500 to 27,000 of the FCoV strain Black (GenBank accession number: EU186072.1) was used to design the RT-LAMP primers used in this study. A set of six primers, including two outer primers (forward primer F3 and backward primer B3), two inner primers (forward inner primer FIP and backward inner primer BIP), and two loop primers (forward loop primer LoopF and backward loop primer LoopB) were selected as the target sequence (Fig. 1 and Table 1).

Detection of FCoV was performed using RT-LAMP. Two different commercial reaction mixtures (Isothermal Mastermix by OptiGene Ltd., UK, PCRun™ molecular detection mix by Biogal, Israel,) were compared using the same set of primers.

For the amplification following the Isothermal Mastermix protocol, the total volume of 25 µl per reaction tube included 15 µl Isothermal Master Mix, 5 µl template, 5 µl Primer Mix and 0.1 µl SuperScript® III Reverse Transcriptase (Thermo Scientific). The Primer Mix consisted of 5 pmol each of F3 and B3 primers, 20 pmol each of FIP and BIP primers and 10 pmol each of LoopF and LoopB primers. For negative control, 5 µl water were added instead of 5 µl template. The reaction mix was incubated at 62 °C for 75 min in a 7500 Real-Time PCR System (Applied

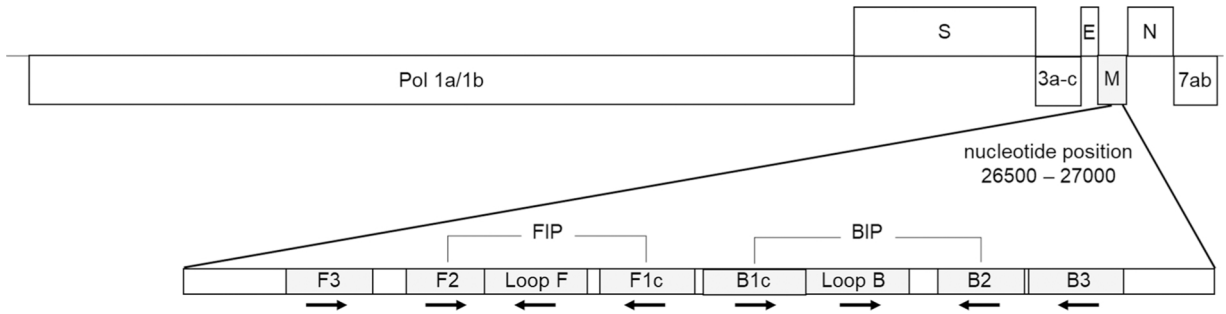

Fig. 1. Position and orientation of RT-LAMP primers. The upper part shows the genomic organization of the FCoV genome. In the lower part the positions of the oligonucleotides used as LAMP-primers in the gene of the membrane protein (M) are shown. Pol 1a/1b,: Polymerase 1a and 1b gene; S, spike protein gene; 3a-c, gene cluster 3abc; E, envelope protein gene; N, nucleocapsid protein gene; 7ab, gene cluster 7ab.

Table 1
Oligonucleotide primers used in the RT-LAMP reaction.

Primer Name	Genome position[a]	Sequence (5'→3')
F3	26695-26715	TGAAGGTTTTAAAATGGCTGG
B3	26774-26795	TCATGTTCACTCAAATTATCAGT
FIP (F1c-F2)	26670-26692 26624-26642	CCAACCAATGTGTAAACGATGGT- CCATCGAGCATTTGCCTAA
BIP (B1c-B2)	26695-26719 26751-26772	AACAATTAAAAGCAACTACTGCCAC- GTGCTTCTGTTGAGTAATCAC
LoopF	26642-26666	ACTAGGTGTAGCAATCATGACGTAT
LoopB	26720-26743	GGGATGGGCTTACTATGTAAAATCT

[a] based on FCoV strain Black (GenBank accession number: EU186072.1).

Biosystems). During RT-LAMP, fluorescence of DNA products was measured once every minute (FAM detection channel, λ_{max}518 nm), and the time to threshold crossing was analyzed. A positive sample (positive in RT-PCR and sequenced for mutations) was included in every run. All samples run in the 7500 RealTime PCR system were subjected to a melt curve analysis after the run. A single sharp peak in the melt curve analysis demonstrates amplification of a single PCR product. The positive samples all showed a single peak and the same melting temperature as the positive control sample.

Following the PCRun™ molecular detection protocol the reaction mix contained 7.5 µl of luminescent reagent, 5 µl of template, 5 µl of Primer Mix and additional 0.1 µl SuperScript III Reverse Transcriptase (Thermo Scientific). The Primer Mix consisted of 7.5 pmol each of F3 and B3 primers, 30 pmol each of FIP and BIP primers and 15 pmol each of LoopF and LoopB primers. For negative control, 5 µl water were added instead of 5 µl template. The reaction mix was incubated at 61 °C for 90 min in a PCRun™ Reader (Biogal). Amplification was detected by measuring bioluminescence twice every minute (Fig. 2).

PCR products of both methods were verified on an agarose gel showing a typical pattern of multiple bands of different molecular weights (Fig. 3).

The results of the 71 samples in the FCoV RT-LAMP assays using two different commercial reaction mixtures are shown in Table 2. Two samples tested false positive using the Isothermal Mastermix and one sample tested false positive with the PCRun™ molecular detection Mix. Sensitivity and specificity for the reported sample groups of both RT-LAMP assays are shown in Table 3. The PCRun™ molecular detection Mix performed better both in sensitivity and specifity than the Isothermal Mastermix. The amplification times for the Isothermal Mastermix positives ranged between 4 and 39 min and for the PCRun™ Molecular Detection Mix positives between 18–77 min.

In the present study, RT-LAMP assays were evaluated as a diagnostic tool for detection of FCoV, in order to distinguish cats with and without FIP that are presented with body cavity effusions. Time from sample to result was kept to a minimum by isolating RNA in about 10 to 15 min using a simple RNA extraction kit. Both commercially available reaction mixtures allowed an easy and fast preparation of the amplification reaction. With LAMP assays, direct detection methods built into the amplification device are preferred, since opening LAMP reaction tubes after amplification is not advisable to decrease the risk of carry-over contamination (Parida et al., 2008; Zanoli and Spoto, 2013). In the present study, a portable device results was used for the PCRun™ Molecular Detection Mix. Positive and negative amplification reactions were indicated by the device with '+' and '-', making it compatible as a point-of-care instrument. The Isothermal Mastermix is intended for use with a portable device, which was not part of this study. The machine used instead replicates the reaction conditions with a constant block temperature and uses a comparable fluorescence detection system.

Both assays tested have similar demands concerning handling skills and preparation time. Detection of positive samples took about half the time with Isothermal Mastermix compared to the PCRun™ Molecular Detection Mix, yet both tests require less than 90 min. The specificity of both methods for the reported sample group was comparable, with 94.6% and 97.3%, respectively. However, false positive results in a test that diagnoses a fatal disease are very critical and should not occur. A cross-reaction of the LAMP-Primers or carry-over contamination might be the cause of these false positive results. However, the negative controls without sample material did not show any indication for carry-over contamination and stayed negative in the LAMP assays. Another possibility for false positive results is the detection of FCoV in cats without FIP, since systemic spread of FCoV does occur, but does not inadvertently result in FIP (Porter et al., 2014). This explanation is not very likely for the three samples of cats without FIP that were positive by RT-LAMP, since all three were negative by RT-PCR. The sensitivity of the PCRun™ Molecular Detection Mix was superior for the reported sample group with 58.8% compared to 35.3% of the Isothermal Mastermix, using the same primers and PCR conditions.

Since the samples for the RT-PCR and for the RT-LAMP had the same preanalytical treatment (frozen, thawed, and centrifuged), the results can be directly compared and showed that the RT-PCR for FCoV performed much better than the RT-LAMP in our sample group. This is in agreement with a study on other coronaviruses, where RT-LAMP also exhibited a lower analytical sensitivity compared to RT-PCR (Bhadra et al., 2015). False negative results can occur in samples with a lower viral burden. The FCoV viral load determined by RT-PCR in effusions has been found to be quite low in some samples of FIP-suspected cats (Lorusso et al., 2017). In our study, the results for RT-PCR were only returned as 'positive' or 'negative' without quantification, leaving open the question whether only effusions with a high viral load resulted in a positive RT-LAMP detection. Another possible reason for the lack of sensitivity might be that sequences of current FCoV strains show sequence variations compared to the sequences deposited in the GenBank database, which were used to design the RT-LAMP primers. Although the primers were chosen to bind in highly conserved regions, variations can occur, which might impair binding and eventually lead to low or no amplification, resulting in poor sensitivity. Reliable primers for RT-PCR target the 3′ UTR of the FCoV sequence, but the RT-LAMP primers that

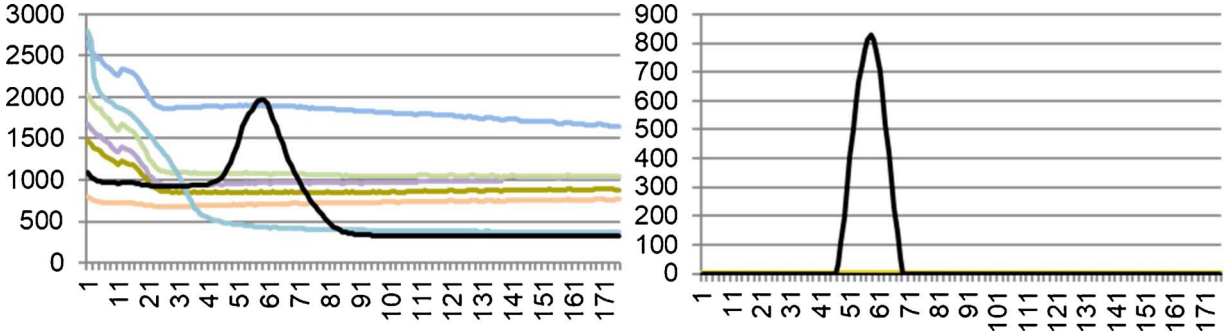

Fig. 2. Detection of a FCoV-positive sample (black) and six FCoV-negative samples by bioluminescence with the PCRun™ Reader. Left panel: raw data, right panel: processed data after background subtraction. Y-Axis luminescence intensity (arbitrary units), X-Axis number of readings (two per minute).

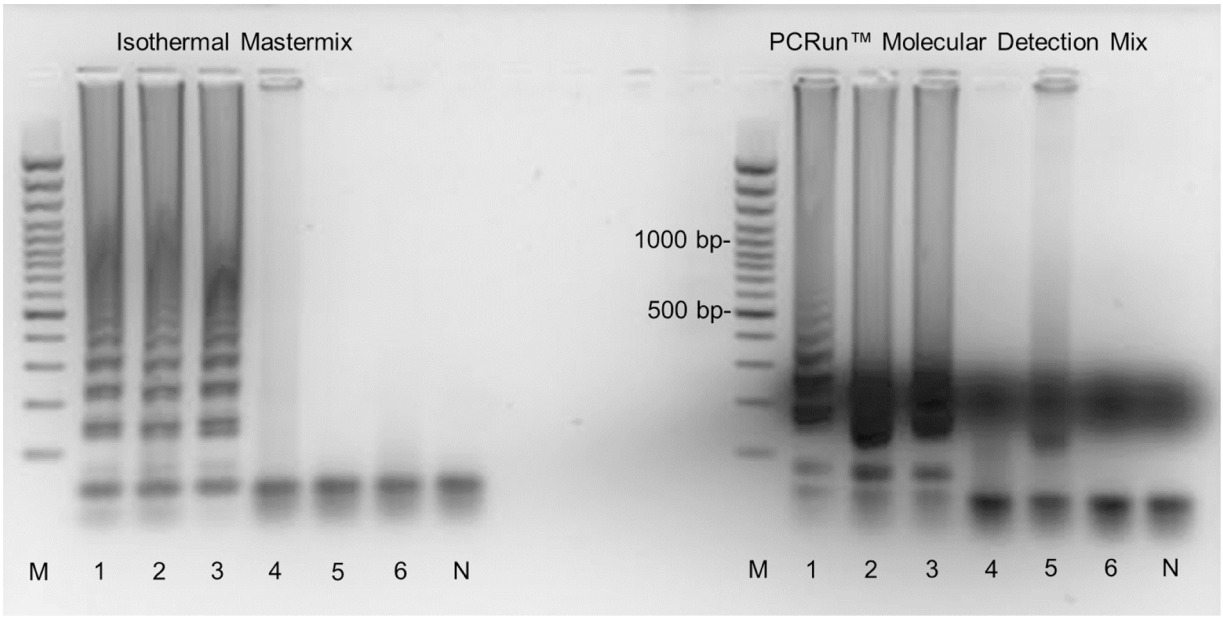

Fig. 3. Detection of FCoV by RT-LAMP with 2% agarose gel electrophoresis. M: 100 bp DNA ladder marker, 1 – 3: FCoV positive samples, 4 – 6: FCoV negative samples, N: negative control samples, either amplified by Isothermal Mastermix or PCRun™ Molecular Detection Mix.

Table 2
Results of the RT-LAMP assays conducted with Isothermal Mastermix and PCRun™ Molecular Detection Mix.

	Isothermal Mastermix		PCRun™ Molecular Detection Mix	
	Positive	Negative	Positive	Negative
FIP	12	22	20	14
Not FIP	2	35	1	36

Table 3
Sensitivity and specificity for the reported sample groups with a prevalence of FIP of 50% of the RT-LAMP assays to diagnose feline infectious peritonitis (FIP) by both Isothermal Mastermix and PCRun™ Molecular Detection Mix.

	Isothermal Mastermix	PCRun™ Molecular Detection Mix
Sensitivity	35,3%	58,8%
Specificity	94,6%	97,3%

were suggested by the PrimerExplorer software in this region included more sequence differences than the M region that we selected. Modifications of the primers might enhance binding and improve sensitivity.

Two studies on LAMP-based identification of FCoV have been published to date. A study from Thailand tested 63 samples of body cavity effusions from cats that were suspected to have FIP both by RT-PCR and by RT-LAMP. More samples tested positive with the RT-LAMP than with the RT-PCR (44% vs. 38%) (Techangamsuwan et al., 2013). However, the inclusion criteria for cats to be suspected of having FIP were not described in that study. Their control samples consisted of plasma and fecal samples from healthy cats without any contact to other cats. The samples from this healthy control group also had more positive results by RT-LAMP than by RT-PCR (50% vs 30%). The authors mention high rates of false positives in the negative controls (no template controls) when using RT-LAMP, so it remains unclear whether their RT-PCR was less sensitive or their RT-LAMP was prone to unspecific amplification. In the second study, different sample types of cats with a clinical suspicion of FIP and fecal samples for screening for FCoV-shedding cats were tested (Stranieri et al., 2017). In most sample types, including effusions, their RT-PCR had about twice as many positive results as their RT-LAMP, and none of the RT-PCR-negative samples was positive in the RT-LAMP method. In agreement with our findings, the sensitivity of the RT-LAMP appears to be inferior to the RT-PCR. For detection of amplification, both studies used gel electrophoresis and one study (Stranieri et al., 2017) additionally used detection of color change from violet to blue with hydroxynaphtol blue. While gel electrophoresis is quite time-consuming, the color change can be difficult to detect in samples with low amplification. Amplification detection with fluorescence or luminescence as used in the present study is preferable, since the results can be read immediately and could be easily converted to a quantitative format with a standard curve. As a perspective for the future, RT-LAMP reactions for virus detection could be run on new devices that integrate nucleic acid extraction, amplification and detection in a miniature format to achieve a true point-of-care diagnosis (Stumpf et al., 2016).

In conclusion, the RT-LAMP in the present study was relatively specific but not very sensitive. The sample type was restricted to effusions of cats unequivocally diagnosed with FIP and of cats without FIP but with clinical signs indicative of FIP. This is a realistic setting in which a veterinarian would use a test for detection of FCoV in the effusion sample. The RT-LAMP assay with the PCRun™ Molecular Detection Mix can be used in a clinical setting to a certain extent. However, before it can replace conventional RT-PCR methods, sensitivity and specificity have to be enhanced by optimizing primers and amplification conditions.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.03.003.

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